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Effects of mGluII Receptor Antagonist LY341495 on Nicotine-Sensitized Rats

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Effects of mGluII Receptor Antagonist LY341495 on Nicotine-Sensitized Rats

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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Abstract

Cigarette smoking has become a worldwide problem leading to millions of fatalities every year. Although most smokers wish to quit, the current smoking cessation methods seem to be ineffective. In order to better understand the addictive qualities of cigarettes, researchers are starting to unravel the neurobiology behind nicotine dependence. The present study evaluated the role of glutamate in nicotine sensitization by measuring both locomotor and brain activities in nicotine-sensitized rats given the novel Group II metabotropic glutamate receptor antagonist LY341495. Subjects treated with LY341495 were expected to show a rise in locomotor activity since the drug was predicted to facilitate glutamate transmission, which would augment the effects of nicotine. However, results show that rats treated with the drug did not show a rise in locomotor activity. In addition, brain activity was expected to be seen in regions associated with the reward pathway (nucleus accumbens, amygdala, ventral tegmental area) and the basal ganglia after administration of LY341495. Results from fMRI appeared to show slight activation in the nucleus accumbens and amygdala but no apparent activity in the ventral tegmental area or basal ganglia. Furthermore, LY341495-treated rats appeared to have decreased brain activity compared to controls which, contrary to the initial hypothesis, could possibly be attributed to the drug's ability to block the effects of nicotine. Thus, to better understand the effects of glutamate and LY341495, the present study should be repeated with an increased dosage of the drug and a greater sample size for imaging.

Table of Contents

Signature Page.....	1
Abstract.....	2
Table of Contents.....	3
Acknowledgements.....	4
Introduction.....	5
Methods.....	18
Results.....	24
Discussion.....	34
References.....	42
Appendix.....	47

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Introduction

According to the American Heart Association, there are 24.8 million men (23.1%) and 21.1 million women (18.3%) smokers in the United States alone. Cigarette smoking has become a worldwide problem and is now the main preventable cause of death. Tobacco use can lead to fatalities resulting from cancer, heart disease, stroke, and lung disease. In fact, smoking causes approximately 443,000 deaths annually in the United States alone and more than 5 million deaths worldwide. Although about 70% of smokers wish to quit, the current cessation methods are ineffective. Those who try to quit often experience depression-like symptoms as well as anxiety, cravings, irritability, mild cognitive impairments, and even physical ailments (CDC Fact Sheet, 2011). These negative withdrawal symptoms are what often cause individuals to relapse, with rates as high as 80% in the first year. Although there are over 4,000 chemicals in cigarettes with 51 of them carcinogenic, nicotine is the main ingredient responsible for addiction (Markou, 2007). In order to fully understand the addictive qualities of cigarettes, researchers are now starting to unravel the neurobiology behind nicotine dependence.

Effects of Nicotine on the Brain

Acute administration of nicotine results in mild feelings of euphoria and slight cognitive enhancement in humans. These positive effects often lead smokers to continue with nicotine use. At this stage, the chronic nicotine state, neuroadaptations have occurred in response to persistent exposure to this psychostimulant (see Figure 2) (Markou, 2008). Similar to other drugs of abuse, nicotine is known to affect the mesocorticolimbic dopaminergic pathway, which is associated with the reward pathway in the brain (see Figure 1). This system consists of dopamine neurons

from the ventral tegmental area (VTA) projecting to the nucleus accumbens (NAcc), amygdala, and prefrontal cortex. The activity of dopamine neurons in the VTA is regulated by glutamatergic projections from the prefrontal cortex, cholinergic inputs from brainstem nuclei, as well as inhibitory inputs from GABA neurons in the VTA and nucleus accumbens (Xi *et al*, 2009).

When nicotine enters the brain, it has two main routes of action: 1) nicotine can bind to the nicotinic acetylcholine receptors (nAChRs) of the $\alpha 4\beta 2$ subtype (higher affinity) on dopaminergic neurons to directly stimulate the release of dopamine into the nucleus accumbens or 2) it can bind to the nAChRs of the $\alpha 7$ subtype (lower affinity) on glutamatergic neurons to trigger the release of glutamate (see Figure 1). This neurotransmitter then interacts with the glutamate receptors on the postsynaptic dopamine neuron, opening the ion-gated channels of the ionotropic glutamate receptors, which induces firing and results in the release of more dopamine into the nucleus accumbens. Aside from stimulating the release of glutamate and dopamine, nicotine also binds to the nAChRs on GABAergic neurons to stimulate the release of GABA, the main inhibitory neurotransmitter in the brain (Markou, 2008). Since GABAergic neurons in the nucleus accumbens have dopamine receptors on the surface, firing of dopaminergic neurons in the VTA to the nucleus accumbens will result in dopamine binding to the GABAergic neurons, which would then cause the release of GABA back to the VTA dopaminergic neurons (Xi *et al*, 2009).

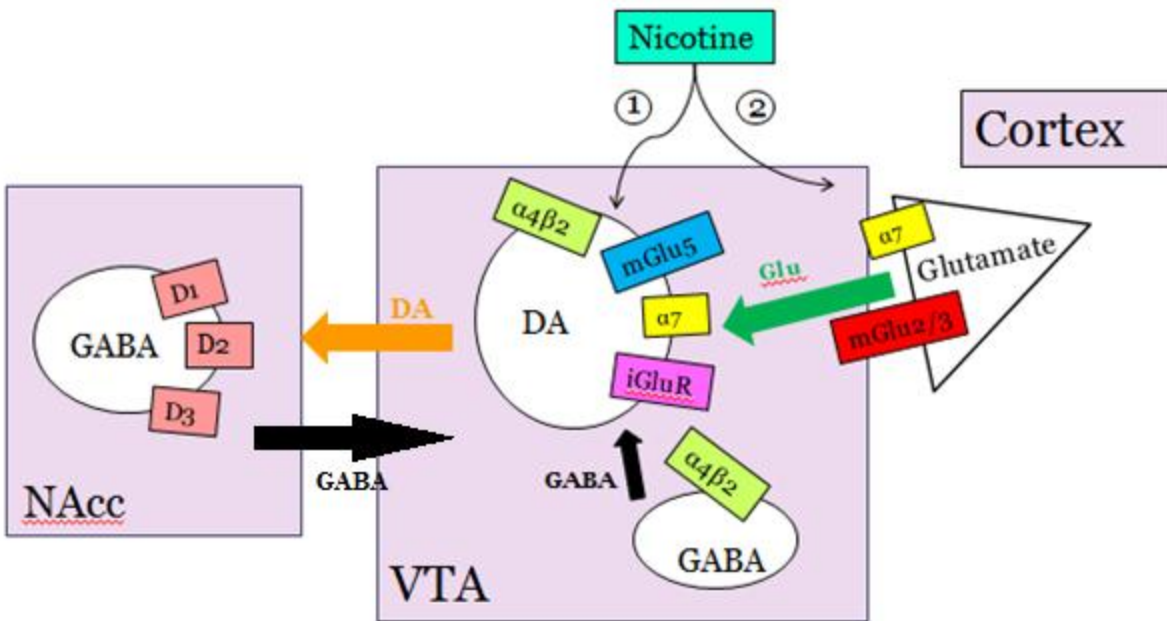


Figure 1: Nicotine's Effects on the Brain

Nicotine has two main routes of action in the brain: 1) binding to $\alpha 4\beta 2$ nAChRs on DAergic neurons in brain sites such as the VTA to directly stimulate release of DA into the NAcc or 2) binding to the $\alpha 7$ nAChRs on glutamatergic neurons to trigger the release of glutamate which then causes increased firing of the DAergic neuron and release of more DA into the NAcc (Xi *et al*, 2009). The small rectangles represent various receptors (D1-D3 represent DA receptors), bolded arrows show the directional transmission of the respective neurotransmitter, large ovals symbolize GABAergic or DAergic neurons, and the triangle represents a glutamatergic neuron.

Nicotinic Acetylcholine Receptors

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels found in various parts of the body. Neuronal nAChRs consist of five subunits combined with a stoichiometry of two α - and three β -, or five $\alpha 7$ -subunits. Found on presynaptic terminals as well as on postsynaptic cells, nAChRs of both $\alpha 4\beta 2$ and $\alpha 7$ subtypes are the most prominent in the brain. As mentioned in the previous section, when nicotine binds to the excitatory nAChRs on presynaptic glutamatergic terminals, it causes an increase in glutamate transmission which then also results in an increase in dopamine transmission – partly due to the activation of postsynaptic nAChRs which causes an influx of Ca^{++} and increased excitability of the cell (Xi *et al*, 2009).

However, in the absence of nicotine, acetylcholine is the endogenous neurotransmitter that binds to and activates the nAChRs (see Figure 2a). Studies have shown that nAChRs of the $\alpha 4\beta 2$ subtype have a high affinity for nicotine and thus, are critical mediators of nicotine's rewarding effects. In rats, nicotine self-administration was ceased when subjects were given the $\alpha 4\beta 2$ receptor antagonist dihydro- β -erythroidine (DH β E) (Watkins *et al*, 1999). In addition, genetic deletion of $\alpha 4$ or $\beta 2$ subtypes inhibited nicotine-generated increases in dopamine levels in the nucleus accumbens (Picciotto *et al*, 1998; Marubio *et al* 2003). Receptors of the $\alpha 4\beta 2$ subtype have also been shown to be present in the majority of nicotine's binding sites in the adult brain: brain slice analysis of $\alpha 4$ - or $\beta 2$ -subunit knockout mice were devoid of high-affinity nicotine binding, indicating that most binding sites for nicotine contain receptors of the $\alpha 4\beta 2$ subtype (Picciotto *et al*, 1998; Picciotto *et al*, 1995).

Aside from receptors of the $\alpha 4\beta 2$ subtype, research has revealed that nAChRs of the $\alpha 7$ subtype also play a role in nicotine's rewarding effects. Similar to the $\alpha 4\beta 2$ nAChRs, $\alpha 7$ -containing receptors are believed to be found on dopamine neurons in the VTA as well. In fact, one study showed that midbrain neurons in $\beta 2$ -subunit knockout mice were still activated by nicotine through interactions with $\alpha 7$ nAChRs (Wooltorton *et al*, 2003). Despite the presence of both receptor subtypes in the VTA, differences in distribution do exist, with $\alpha 4\beta 2$ subtypes found predominantly on GABAergic terminals – though receptors of this subtype are also located on dopaminergic neurons – while $\alpha 7$ subtypes are found mainly on glutamatergic terminals (see Figure 1) (Xi *et al*, 2009).

Frequent and recurring administration of nicotine can lead to rapid desensitization of $\alpha 4\beta 2$ nAChRs, which then results in upregulation and increased expression of this receptor type on the cell surface. Evidence suggests that this nicotine-induced phenomenon is due to a protein

kinase C (PKC)-dependent pathway which involves the phosphorylation of immature $\alpha 4$ subunits on serine residues, resulting in receptor maturation and assembly (Wecker *et al*, 2010). Due to the differences in degrees of desensitization and affinity, it has been suggested that nicotine first interacts with and desensitizes $\alpha 4\beta 2$ nAChRs (high affinity), while the lower affinity and desensitization rate of $\alpha 7$ nAChRs, along with instigating the release of glutamate, will extend the activation time of dopamine neurons (Xi *et al*, 2009).

Aside from nAChR upregulation, chronic exposure to nicotine can also result in behavioral sensitization: frequent and recurring administration of nicotine causes long-term enhancement of dopaminergic and behavioral activity to the point where re-exposure to nicotine (even after weeks and months) will cause stronger dopaminergic and behavioral responses than observed initially. Therefore, behavioral sensitization is a result of nicotine-induced nAChR upregulation followed by long-term potentiation of excitatory inputs to dopamine neurons (Vezina *et al*, 2007). Numerous studies have been conducted on the relationship between nicotine and the dopamine system, but attention now is being directed to the glutamate system. More specifically, metabotropic glutamate receptors (mGluR) are now seen as potential targets for smoking cessation therapies.

Glutamate

In the mammalian central nervous system, glutamate is the major excitatory neurotransmitter and a contributor to the effects of nicotine. Glutamatergic terminals can be found in areas such as the VTA, nucleus accumbens, prefrontal cortex, and hippocampus. Glutamatergic afferent projections to areas such as the VTA, nucleus accumbens, and other brain

sites that contain dopaminergic cells or terminals originate from regions such as the frontal cortex, amygdala, and hippocampus (Markou, 2007).

Both ionotropic (iGlu) and metabotropic glutamate (mGlu) receptors regulate the transmission of glutamate. Ionotropic glutamate receptors are mainly located postsynaptically and include NMDA (*N*-methyl-D-aspartate), AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate), and kainate receptor subtypes (Markou, 2007). When activated, these receptors increase the cellular excitability by opening the glutamate-gated ion channels, allowing an influx of Na^+ and Ca^{++} ions and outflow of K^+ ions, which results in depolarization and firing of the postsynaptic cell (Xi *et al*, 2009).

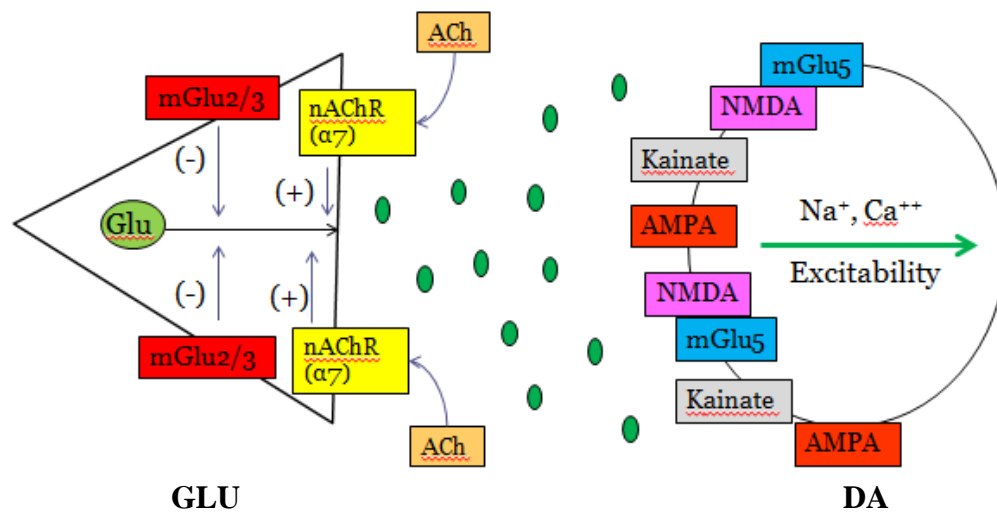
On the other hand, there are currently eight known mammalian subtypes of metabotropic glutamate receptors, and they have been classified into three groups (I, II, III) based on sequence homology, signal transduction pathways, and pharmacological selectivity. Metabotropic glutamate receptors are expressed in numerous areas throughout the brain and are slower acting compared to the ionotropic glutamate receptors. Group I receptors are mainly located postsynaptically and consist of mGlu1 and mGlu5 receptors. They couple to G-proteins to activate phospholipase C, and they also couple to intracellular Homer proteins that are vital for transporting mGlu receptors in and out of synapses. Depending on the situation, Group I receptors can be excitatory or inhibitory. Group II receptors are inhibitory autoreceptors found primarily presynaptically and consist of mGlu2 and mGlu3 receptors. They serve to negatively regulate the release of glutamate by presynaptic inhibition: glutamate released from the glutamatergic terminal binds to these inhibitory Group II receptors. Group II receptors couple to G-proteins to negatively regulate adenylyl cyclase activity – inhibiting the creation of the second messenger for signal transduction cAMP. Group III receptors are also mainly situated

presynaptically and consist of mGlu4, mGlu6, mGlu7, and mGlu8 receptors. They also couple to G-proteins but to decrease adenylyl cyclase activity (Markou, 2007).

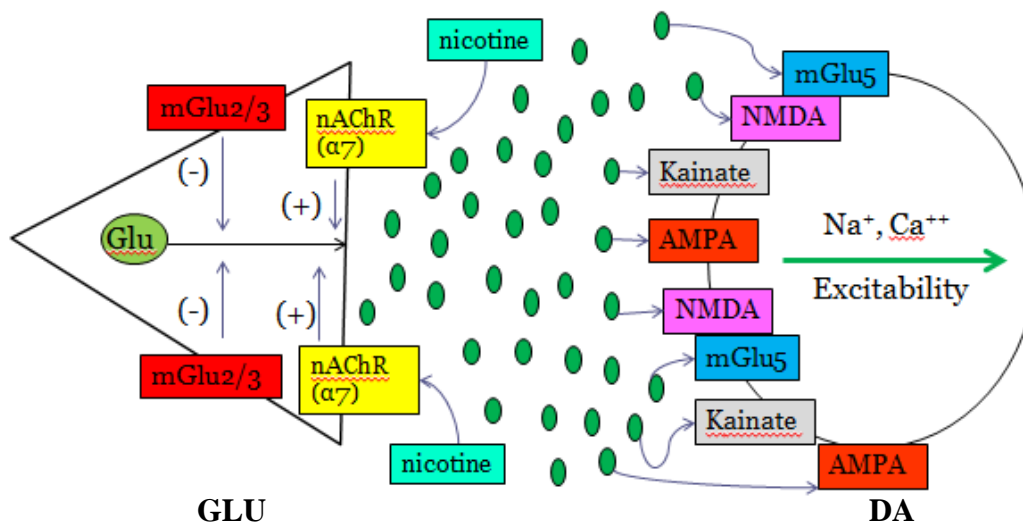
Since disruption in glutamate transmission has been linked to psychiatric disorders such as depression, anxiety, schizophrenia, and addiction, altering the activity of mGlu receptors has been suggested as a possible treatment for these illnesses (Chaki *et al*, 2003). Given that mGlu receptors can be found throughout the brain, altering glutamate transmission in pharmacologically subtle ways would presumably affect motivated behavior without creating unwanted or even toxic side-effects (Conn and Pinn, 1997). In fact, a study conducted on an animal model of schizophrenia, known as the phencyclidine model, showed that the administration of an mGluII receptor agonist resulted in behavioral reversals such as improved working memory and locomotion. These behavioral improvements were seen despite continued dopamine hyperactivity in the subjects, providing evidence that dopamine does not have to be involved in treatments for psychiatric disorders (Moghaddam and Adams, 1998).

The present study focused on Group II metabotropic glutamate (mGluII) receptors (mGlu2 and mGlu3). As mentioned earlier, the binding of nicotine to excitatory nAChRs on presynaptic glutamatergic terminals will cause an increase in glutamate transmission. As a result of the rise in levels of glutamate in the synapse, inhibitory mGluII receptors upregulate their activity to restore glutamate to pre-nicotine levels – signifying the chronic nicotine state. Thus, when smokers cease to smoke, glutamate transmission is decreased due to the increased activity of the inhibitory mGluII receptors (see Figure 2). This would then lead to a reduction in dopamine transmission which is often the cause for nicotine cravings and the associated negative withdrawal symptoms (Markou, 2007).

2a) Nicotine Naïve State



2b) Acute Nicotine State



2c) Chronic Nicotine State

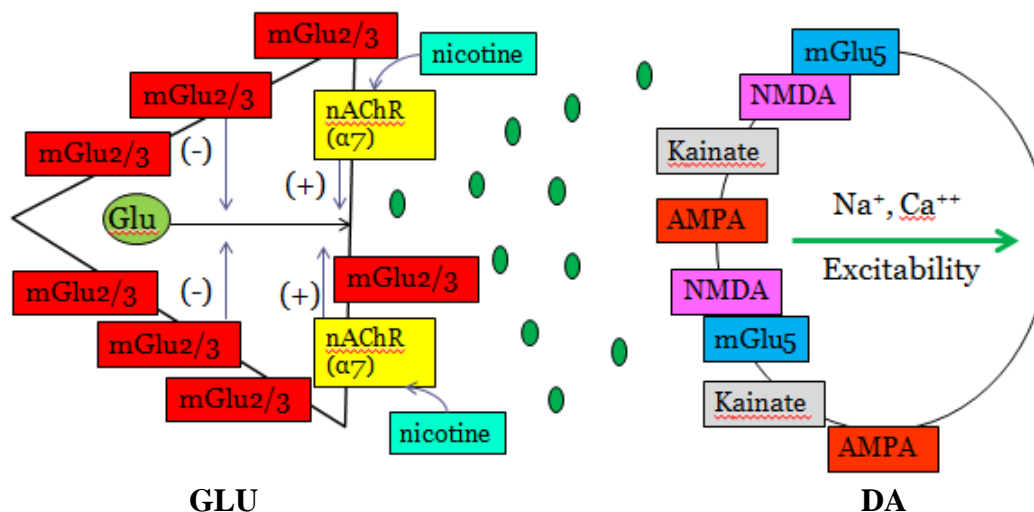


Figure 2: Nicotine's Effects on Glutamate Transmission

The triangle on the left represents the glutamatergic neuron and the oval on the right represents the postsynaptic DA neuron. The small rectangles symbolize acetylcholine, nicotine, or various presynaptic and postsynaptic receptors while the green ovals represent glutamate.

2a) Acetylcholine is the endogenous neurotransmitter that binds to nAChRs in the absence of nicotine and causes glutamate to be released into the synapse. This neurotransmitter then binds to the postsynaptic glutamate receptors to increase cellular excitability, which results in firing of the postsynaptic DA neuron. **2b)** Acute administration of nicotine results in an increase in glutamate transmission which then causes increased firing of the DA neuron and raised levels of DA in the NAcc. **2c)** Since nicotine causes an increased release of glutamate, the inhibitory autoreceptors mGlu2 and mGlu3 (mGluII receptors) increase their activity to restore glutamate to pre-nicotine levels (shown in the figure as an increased number of mGlu2/3 receptors). This signifies the chronic nicotine state and dependence: nicotine is required in order for glutamate levels to be similar to that of the nicotine naïve state (Markou, 2007).

The main subject of this study is the novel mGluII receptor antagonist LY341495. Since it is an antagonist, LY341495 is believed to increase glutamate transmission by binding to the mGluII receptors and preventing their inhibitory activity (see Figure 3). A previous study where no nicotine was involved showed that LY341495 had an antidepressant-like effect seen in the rat forced swim test and mouse tail suspension test (Chaki *et al*, 2003), with the idea that decreased glutamate transmission contributes to the depression-like state often experienced by smokers in

withdrawal. Moreover, LY341495 has also been proven to attenuate the reward deficits – elevations in self-stimulation thresholds measured via intracranial self-stimulation by rats in the chronic nicotine state – which is often associated with nicotine withdrawal (Kenny *et al*, 2003).

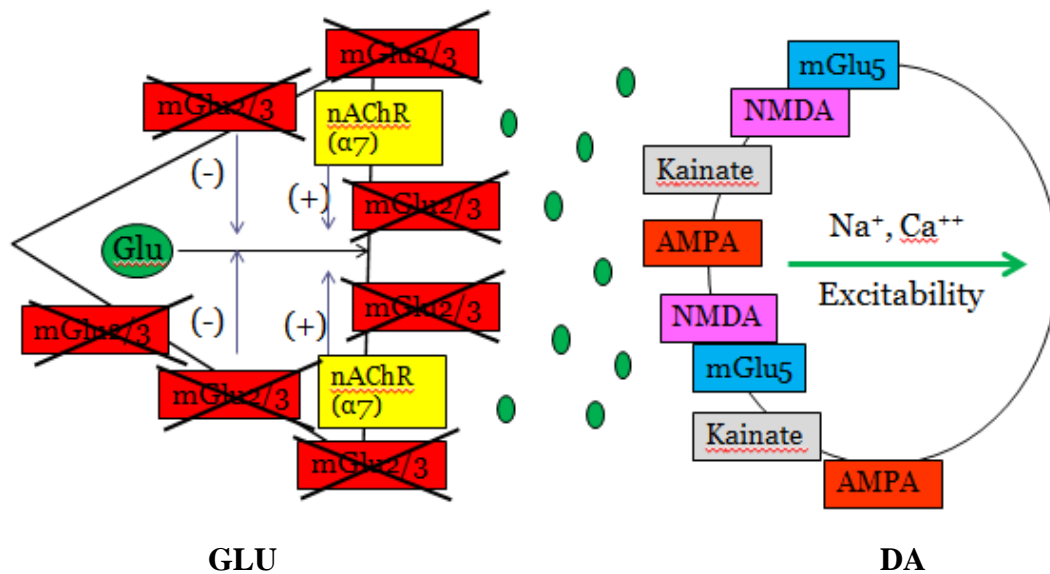


Figure 3: Administration of the mGluII receptor antagonist LY341495 is believed to increase glutamate transmission and restore glutamate to pre-nicotine levels by blocking the inhibitory effects of the mGluII receptors (Markou, 2007).

The goal of the present study is to evaluate the role of glutamate in nicotine sensitization by observing the effects of LY341495 on locomotor and brain activity in nicotine-sensitized rats. In drug studies, neural sensitization occurs when the effects of a drug are increased after repeated administration, whereas tolerance occurs when the effects of a drug are decreased upon repeated administration. However, behavioral sensitization is the process in which the same dosage of drug is given, but the behavioral effects – locomotor activity – gradually increases before reaching a plateau. A model associating the interactions of dopamine, glutamate, and GABA has

been proposed to be involved in the induction and expression of behavioral sensitization (see Figure 4).

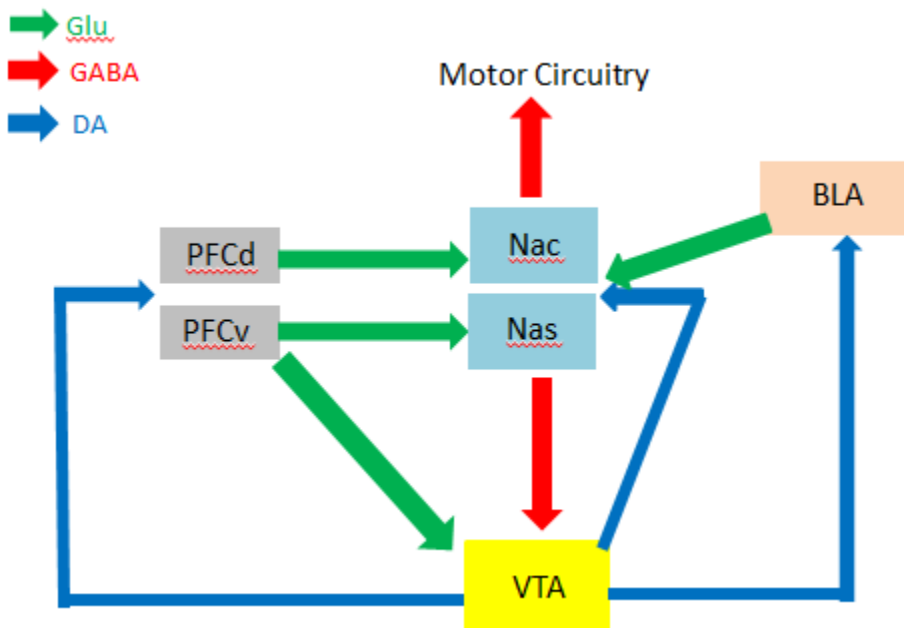


Figure 4: Model of the interaction among Glu, GABA, and DA believed to be involved in the induction and expression of behavioral sensitization (Vanderschuren and Kalivas, 2000). The arrows show the direction of transmission for each respective neurotransmitter. PFCd = dorsal prefrontal cortex (prelimbic and anterior cingulate), PFCv = ventral prefrontal cortex (infralimbic), Nac = nucleus accumbens core, Nas = nucleus accumbens shell, BLA = basolateral amygdala, VTA = ventral tegmental area. The VTA and BLA send signals to both the Nac and Nas (DA and Glu, respectively).

In nicotine naïve rats, the first few doses of nicotine will result in decreased locomotor activity. After repeated exposure to this psychostimulant, the rats will be in the chronic nicotine state (see Figure 2c). Behavioral sensitization to nicotine will also occur, marked by increased locomotor activity. This phenomenon is due to tolerance to nicotine's depressant effects (Collins

et al, 1988) and sensitization to its stimulant effects (Clarke and Kumar, 1983a; Ksir *et al*, 1985) – which can be attributed to stimulation of dopamine neurons (Balfour *et al*, 1998).

The effects of the mGluII receptor antagonist on locomotor activity of nicotine-sensitized rats were assessed by an open-field test which measured the total distance traveled by each subject. Research has shown that reducing glutamate transmission inhibits the rewarding effects of nicotine (Markou, 2008). Since LY341495 is thought to increase glutamate transmission, nicotine-sensitized rats treated with the drug are expected to show a rise in locomotor activity. Results from a separate study where no nicotine was involved show that mice that received subcutaneous injections of LY341495 displayed an increase in locomotor activity (O'Neill *et al*, 2003).

In order to observe the effects of LY341495 in the brain, functional magnetic resonance imaging (fMRI) was used to study localization of brain activity. This non-invasive procedure measures BOLD (blood-oxygenation-level-dependent) response: relative to the resting state, an increase in concentration of oxygenated blood indicates neural activity while an increase in deoxygenated blood indicates neural inactivity (Logothetis, 2002). Previous studies have shown that exposure to cigarette/nicotine-related cues to those dependent – which leads to craving – results in increased activity of the mesolimbic system (right posterior amygdala, posterior hippocampus, VTA, and medial thalamus) (Due *et al*, 2002) as well as increased glucose metabolism in parts of the anterior paralimbic system (anterior cingulate cortex (ACC), posterior orbitofrontal cortex (OFC), and anterior insula), dorsolateral prefrontal cortex (DLPFC) and right superior sensorimotor cortex (Brody *et al*, 2002). Hence, rats are expected to show positive BOLD response in brain regions associated with craving before nicotine administration.

After the injection of nicotine, rats that received the mGluII antagonist are expected to show positive BOLD signal changes in the rat reward pathway – specifically in the prefrontal cortex, VTA, nucleus accumbens, and amygdala – since the increase in glutamate transmission would presumably amplify the rewarding effects of nicotine. In addition, there is evidence that increased activity at glutamatergic projections from the prefrontal cortex to nucleus accumbens enhances cocaine seeking in rats (Moran *et al*, 2005), and the actions of nicotine appear to be very similar to this central nervous stimulant. Finally, activation of the basal ganglia (caudate nucleus, putamen, globus pallidus, subthalamic nucleus, and substantia nigra) is also expected. This brain region plays a vital role in the control of movement and is rich with mGluII receptors (Sacaan et al, 1992; Wright et al, 1994).

Materials and Methods

Animals

Male Sprague-Dawley rats (behavioral test $n=16$: LY341495-treated group $n=8$, control/saline-treated group $n=8$; imaging: LY341495-treated group $n=2$, control/saline-treated group $n=4$) (from Harlan Laboratories) weighing 300-350 g upon arrival in the laboratory were housed two per cage in a temperature- and humidity-controlled room on a 12 hour reverse light-dark cycle (lights off at 9 AM). After arrival, rats were given approximately 5 days to become accustomed to their new environment before any testing. All open-field experiments and imaging were performed during the dark phase of the light-dark cycle. Food and water were readily available (except during testing). A different batch of rats was used in the imaging study (not the same subjects from the open-field test). All experimental procedures were in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

Drugs

(-)-Nicotine hydrogen tartrate was purchased from Sigma (St. Louis, MO) and dissolved in saline, with pH adjusted to 7 with sodium hydroxide. Rats were given 0.4 mg/kg of nicotine immediately before being placed into the open arena. The novel mGluII receptor antagonist LY341495 [(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-[xanth-9-yl]propionic acid] was purchased from Tocris Bioscience Cookson (Ballwin, MO) and dissolved in saline, administered (1mg/kg) via subcutaneous injections in a volume of 1 mL/kg of body weight.

LY341495 was administered 30 minutes prior to the nicotine injection and before placement into the open-field arena.

Open-field arena

Rats were placed into a 90 cm x 90 cm black Plexiglas square box with an open top. Two red light bulbs and a video camera were mounted approximately 3 ft. above. The camera was connected to the video tracking software EthosVision to measure the rats' locomotor activity: the program divided up the arena into squares and measured the amount of time that the rat stayed in each section.

fMRI magnet

A Bruker 4.7T/40cm horizontal magnet with a 20 Gauss/cm magnetic field gradient insert (inner diameter = 12cm, Bruker, Billerica, MA, USA) was used for the imaging studies. Rapid acquisition relaxation enhanced sequence (RARE) with TR (relaxation time) = 2 sec, TE (echo time) = 12 msec, resolution matrix = 256 x 256, FOV (field of view) = 30 mm x 30 mm, eighteen 1.2 mm slices were used to generate high resolution multi-slice anatomical images. With the same FOV and slice thickness at 1800 repetitions, the saline/LY341495 and nicotine scans used echo-planar imaging sequence (EPI) at a resolution of 64 x 64. TR = 1 sec, TE = 30 msec, and each scan ran for a total of 30 minutes. In the first EPI scan, a 1 minute baseline period was allotted before injection of saline (control) or LY341495 (treatment). Afterwards, a second EPI scan also totaling to 30 minutes was performed, with a 1 minute period reserved for baseline before injection of nicotine. Rats were secured into a dual coil restrainer (volume and surface coils) before being placed into the magnet.

Experimental procedures:

Open-field test

Behavioral studies were performed at the same time on all test days. After allowing the rats to become accustomed to their new environment for approximately 5 days, each rat was individually placed into the open arena and its locomotor activity was recorded for a 30 minute session to establish a baseline (Day 0) (see Figure 5). Each rat was given a subcutaneous mock injection 30 minutes prior to being placed into the arena to let them become accustomed to this procedure and reduce stress on the actual treatment days. For the next 5 consecutive days (Days 1-5), each rat was given a mock injection followed by a subcutaneous nicotine injection 30 minutes later and immediately placed into the open arena for a recorded 30 minute session. On Days 6 and 7, the rats were divided into the test group (LY341495-treated) and control group (saline-treated). All procedures were the same as before but instead of a mock injection, rats were either given a SC injection of LY341495 or saline 30 minutes prior to nicotine and testing.

<u>Day</u>						
0	→	OFT				
1	→	Nicotine	→	OFT		
2	→	Nicotine	→	OFT		
3	→	Nicotine	→	OFT		
4	→	Nicotine	→	OFT		
5	→	Nicotine	→	OFT		
6	→	Saline/LY341495	→	Nicotine	→	OFT
7	→	Saline/LY341495	→	Nicotine	→	OFT

Figure 5: Open-Field Test Experimental Procedures

On Day 0 of the open-field test (OFT), no drugs were given and subjects were placed into the open arena in order to establish a baseline locomotor activity. For the next 5 consecutive days (Days 1-5), rats received daily SC injections of nicotine before being immediately placed into the arena. On Days 6 and 7, subjects received either saline (control group) or LY341495 (treatment group) 30 minutes prior to nicotine administration before being placed into the arena.

Imaging

Aside from allowing the rats to familiarize themselves with their new environment for 5 days, a validated acclimation procedure (King *et al*, 2005) was also performed in order to reduce stress for the animals while imaging. A Lidocaine (2.5%) and Prilocaine (2.5%) paste was applied to the ear canals of each rat 30 minutes prior to the acclimation procedure to numb the area and minimize discomfort. Each subject was first lightly anesthetized with isoflurane. Afterwards, a plastic semicircular headpiece with blunted ear supports to fit into the ear canals was placed on each rat's head (on site of Lidocaine and Prilocaine paste application). The head of the rat was placed into a cylindrical head restraint and its incisors were secured over a bite bar. A screw on each side of the head restraint (total of 2) aligned up with the headpiece to secure the rat's head. After being strapped into the head restraint, the animal's body was placed into a custom-fitted cylindrical plastic tube with its limbs taped to reduce motion and prevent the subject from breaking free. Finally, an opaque tube was placed over the head restraint to imitate the darkness of the fMRI magnet. The rat in its body tube and head restraint covered by the opaque tube was then placed next to a speaker in a dark, isolated room playing a recording of the actual scanner noise. This procedure was conducted for 8 days with gradual increases in time exposed to the fMRI recording: 15, 30, 45, 60, 75, and 90 minute sessions (75 and 90 minute sessions were performed twice).

Upon completion of the acclimation procedure (after 8 sessions), rats were given a daily subcutaneous injection of nicotine for 7 consecutive days (Days 1-7). Similar to the open-field test, subjects were also given either saline (control group) or LY341495 (treatment group) 30 minutes prior to the nicotine injection on Days 6 and 7.

Rats were imaged on Day 7 and, similar to the acclimation procedure, they were given Lidocaine (2.5%) and Prilocaine (2.5%) paste 30 minutes prior to anesthesia with isoflurane. A semicircular headpiece was placed over the ear canals, and the rat's head was secured into the head restraint. Since the rat cannot be given a subcutaneous injection while in the magnet, 2 labeled syringes (1 with nicotine and the other with saline or LY341495), with wing needle extensions to allow for injections from outside the magnet, were placed into the rat's back. The subject's body was then placed into the imaging tube (dual coil restrainer) and into the fMRI magnet. Each rat underwent 3 scans that totaled to 108 minutes (see Figure 6): an 8 minute anatomy scan followed by two 30 minute EPI scans (saline or LY341495 and then nicotine). In both the saline (control) and LY341495 (treatment) EPI scans, saline and LY341495 were administered 1 minute after the beginning of the scan in order to establish a baseline for BOLD responses. After the 30 minute long saline or LY341495 scan, the second EPI scan was performed and nicotine was also administered to both the control and treatment groups after one minute in order to establish a baseline.

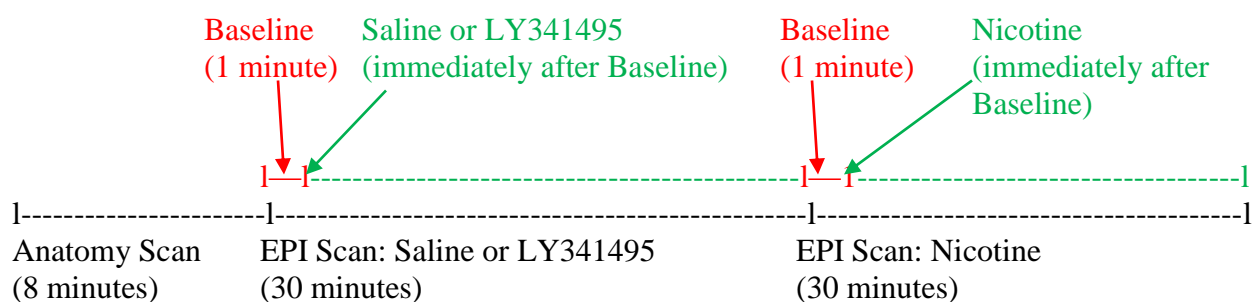


Figure 6: fMRI scans

Each rat underwent 3 scans that totaled to 108 minutes: 1) 8 min. anatomy scan, 2) 30 min. EPI scan where saline (control) or LY341495 (treatment) was injected after obtaining 1 min. baseline, and 3) another 30 min. EPI scan where nicotine was injected after obtaining 1 min. baseline.

Analysis:

Open-field test

Each subject's total locomotor activity (total distance traveled) for all test days was recorded during the open-field test and calculated by EthosVision. Microsoft Excel was then used for statistical measurements: mean, standard deviation, ANOVA (between control and treatment groups as well as within the treatment group).

fMRI

Imaging results were extracted from ParaVision and imported into MIVA to line up brain slices from the anatomical scans of each rat to the standard anatomy/representative and then aligned with a segmented digital rat brain atlas. Afterwards, Matlab (Math-Works Inc., Natick, MA) was used to correct any motion artifact before the data from all rats in a group were averaged. SPM8 (Statistical Parametric Mapping) was then used to generate the resulting BOLD response images.

Results

Since LY341495 is a Group II metabotropic glutamate receptor antagonist, it is believed to block the inhibitory activity of mGluII receptors. As a result, more glutamate can be transmitted which then ultimately leads to increased dopamine transmission. Increasing dopamine transmission is hypothesized to augment the acute effects of nicotine. In the present study, an open-field test was used to measure the effects of LY341495 on locomotor activity in nicotine-sensitized rats. In addition to assessing behavioral changes, fMRI was also conducted to view differences in brain activity localization as a result of LY341495.

Open-field test

Since LY341495 is believed to increase glutamate transmission, nicotine-sensitized rats treated with the drug are expected to show an increase in locomotor activity. To test this hypothesis, an open-field test was used to measure the mean locomotor activity for both the control (receiving saline) and treatment groups (receiving LY341495). The experiment required a total of 8 days, where Day 0 represented the baseline (no LY341495, saline, or nicotine were administered). Afterwards, all subjects received a daily subcutaneous injection of nicotine (Days 1-7), but on Days 6 and 7, the treatment group was also given LY341495 30 minutes prior to the nicotine injection, while the control group was given saline 30 minutes prior to nicotine (see Figure 5).

The mean locomotor activity for both the control and treatment groups can be seen in Figure 7 (see raw data in Appendix). Day 0 represents the baseline – no nicotine, saline, or LY341495 was given. The mean locomotor activity for both the test group ($n=8$) and control

group ($n=8$) were very similar on Day 0. A major drop in locomotor activity was also seen in both groups on Day 1 – when all subjects were given their first dose of nicotine. From Day 1 onwards, both groups showed a general increasing trend in mean locomotor activity. However, aside from Day 0, the control group always had higher average activity compared to the LY341495 group. In fact, significant differences in locomotor activity were seen between the groups on Days 6 and 7. On Days 6 and 7 where the subjects received either saline or LY341495, the control group seemed to reach a plateau while the test group still showed increase in locomotor activity – though not significant – but never reaches past 11,018.32 cm. Overall, the control group had higher mean locomotor activity that increased at a greater rate compared to the test group on all days except on baseline (Day 0) and treatment days (Days 6 and 7). Although these results suggest a difference between treatment and control groups, they are difficult to interpret because the treatment group's mean locomotor activity was consistently lower than the control's mean activity (aside from baseline). Moreover, the differences in locomotor activity (within group) seen on Days 6 and 7 of the treatment group were not statistically significant (compared to each other and Day 5).

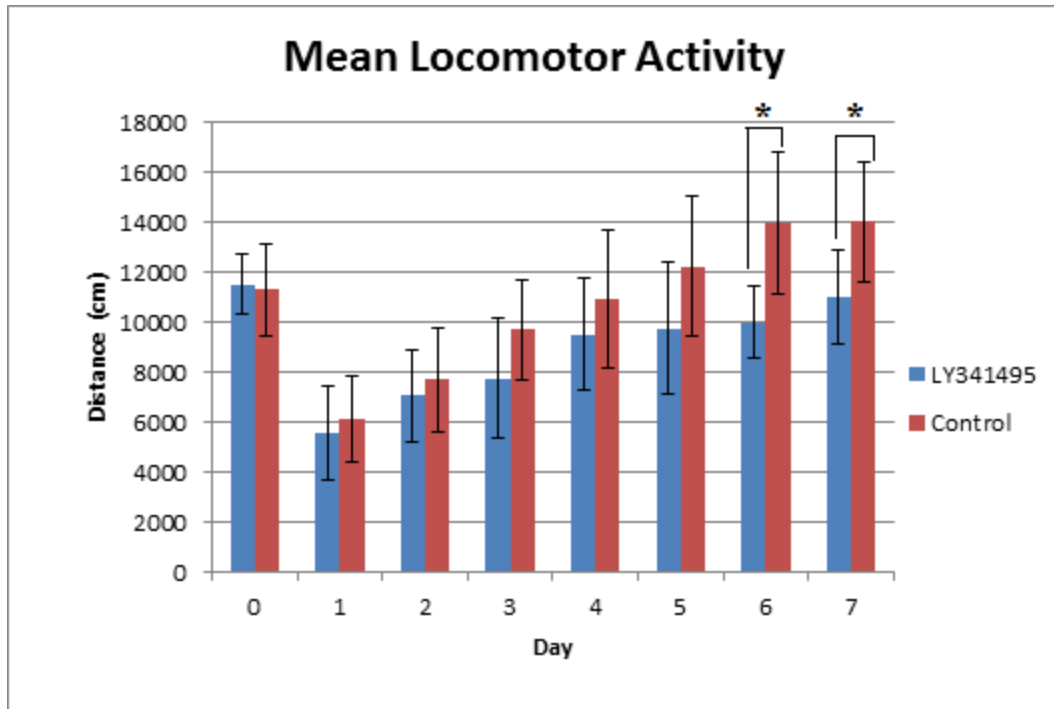


Figure 7: Mean Locomotor Activity from Open-Field Test

The locomotor activity for the control ($n=8$) and LY341495-treated ($n=8$) groups were recorded on all days by EthoVision. The total distance moved (cm) by the subjects in each group were averaged and standard deviations were calculated (capped black lines). Subjects did not receive any nicotine, saline, or LY341495 on Day 0 (baseline), but they did receive nicotine on Days 1-7. On Days 6 and 7, the control group received saline 30 minutes prior to the nicotine injection while the treatment group received LY341495 30 minutes before nicotine. ANOVA ($p < 0.05$) was performed between groups for Days 1-5 and within group for Days 5-7.

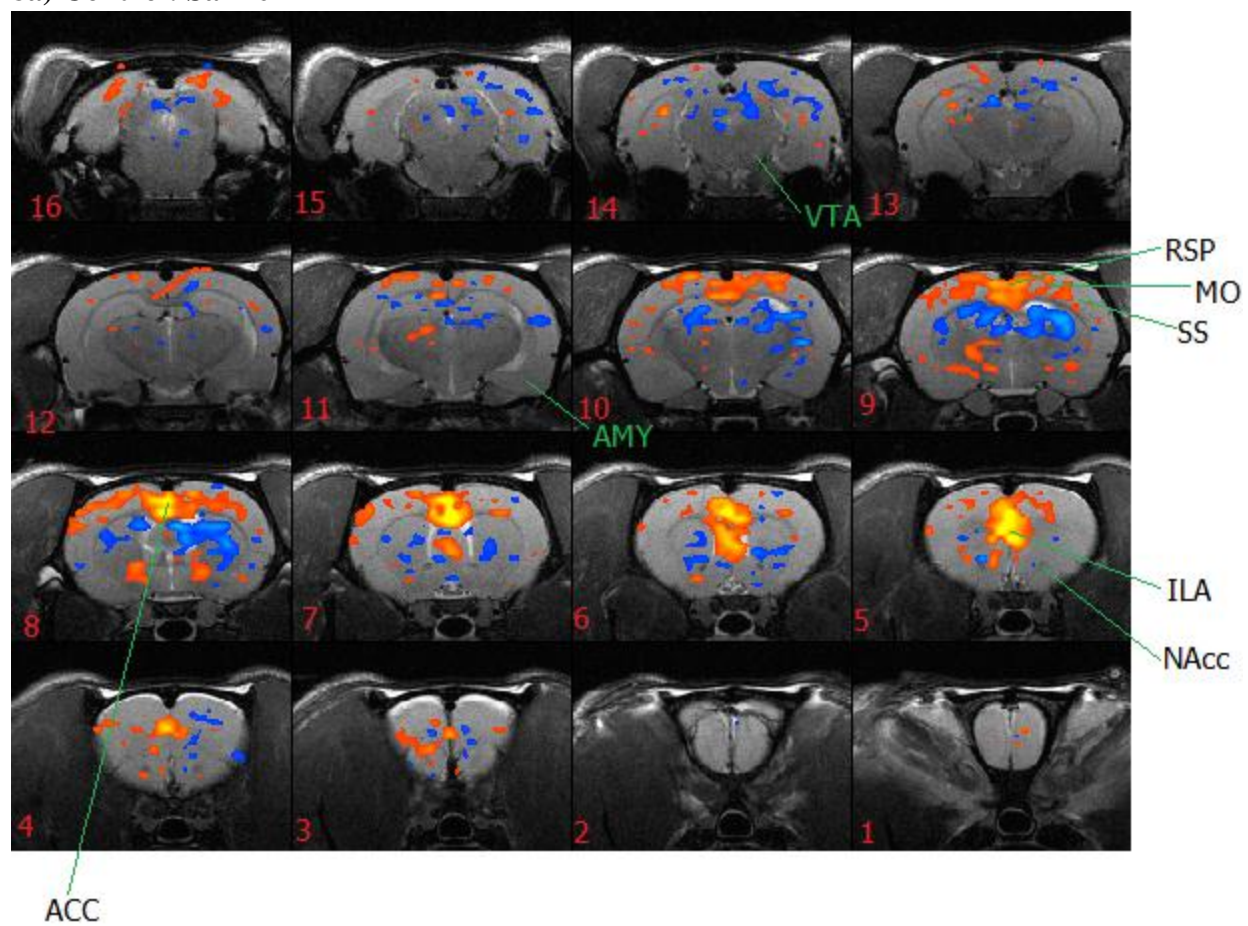
fMRI

After performing the open-field test to measure behavioral differences (locomotor activity), the effects of LY341495 in the brain of nicotine-sensitized rats were also assessed through fMRI. A new batch of rats were used (not the same subjects from the open-field test) but since the imaging study should mirror the behavioral study, the rats were again divided into the control and treatment groups. In order to reduce stress, which would affect the imaging results, all rats underwent an acclimation procedure prior to the imaging session (see Methods for

details). After acclimation, subjects were given a subcutaneous injection of nicotine for 7 consecutive days, similar to the open-field test, and imaging was performed on Day 7. The rats received either LY341495 or saline 30 minutes prior to the nicotine injection on both Days 6 and 7, but on Day 7, the drugs (LY341495 and nicotine) and saline were administered while the rat was in the magnet.

On the imaging day (Day 7), after being properly secured into the head restraint and body tube, the subjects were placed into the magnet for a 108 minute-long scan: an 8 minute anatomy scan and two 30 minute EPI scans (for saline or LY341495 and nicotine). Figures 8 and 9 show the imaging results from the two groups that were gathered through ParaVision and generated from Matlab. The threshold for the images is set at 1.0, which means that differences in blood-oxygen-level below or above 1% are detected and shown. The orange/yellow represents positive BOLD response (increase in concentration of oxygenated blood, signifying neural activation) while the blue represents negative BOLD response (increase of deoxygenated blood, signifying neural inactivation).

8a) Control: Saline



8b) Control: Nicotine

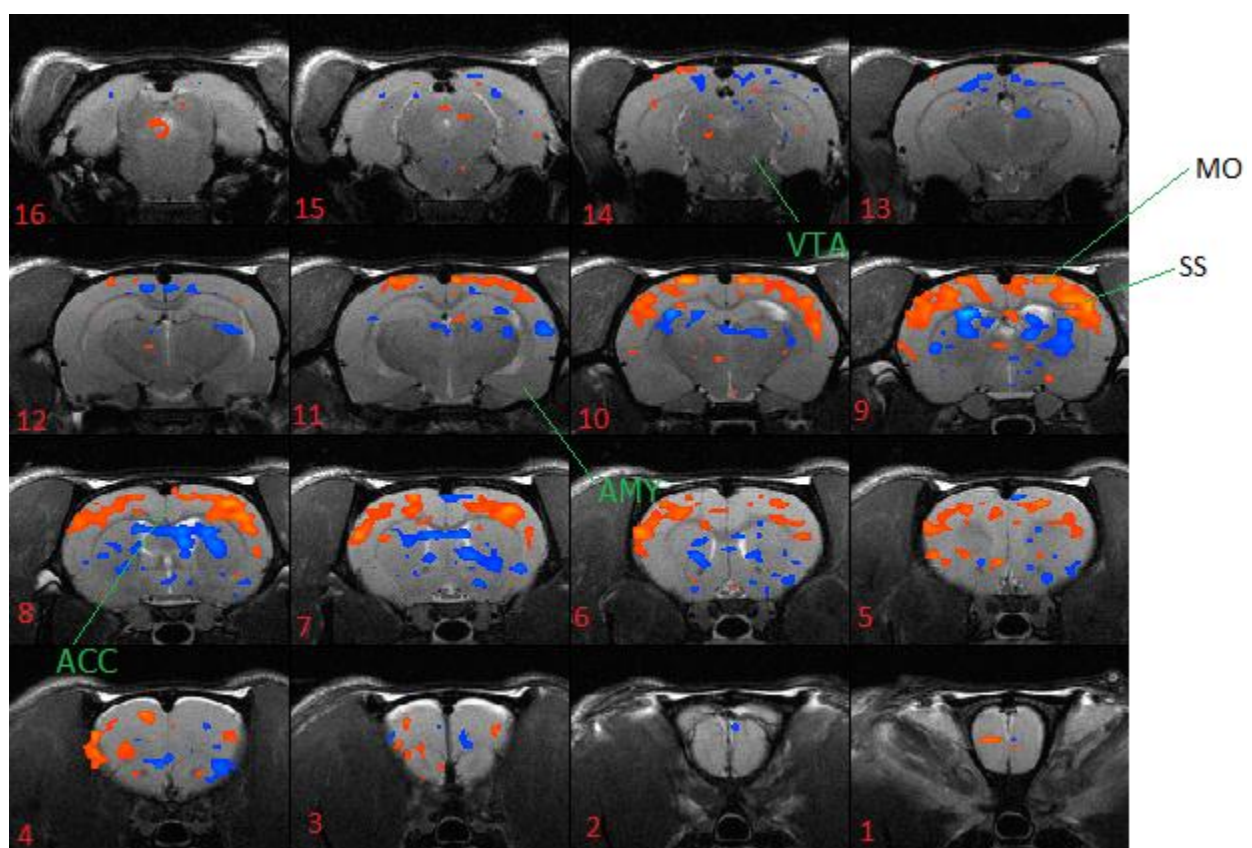


Figure 8: BOLD Response from fMRI of Control Group ($n=4$)

(DISCLAIMER: Images were obtained from a separate nicotine study that followed the same protocol as the present study except a different drug – an NMDA receptor antagonist CGP39551 – was used. The control group received the same treatment and drugs as the control group in the present study.) After the initial anatomical scan, two 30 minute EPI scans were performed (after injection of saline and then after nicotine). The 16 images are brain slices from the forebrain to the hindbrain in numerical order. (MO = somatomotor area, SS = somatosensory area, ACC = anterior cingulate cortex, NAcc = nucleus accumbens, ILA = infralimbic area, RSP = retrosplenium, AMY = amygdala, VTA = ventral tegmental area)

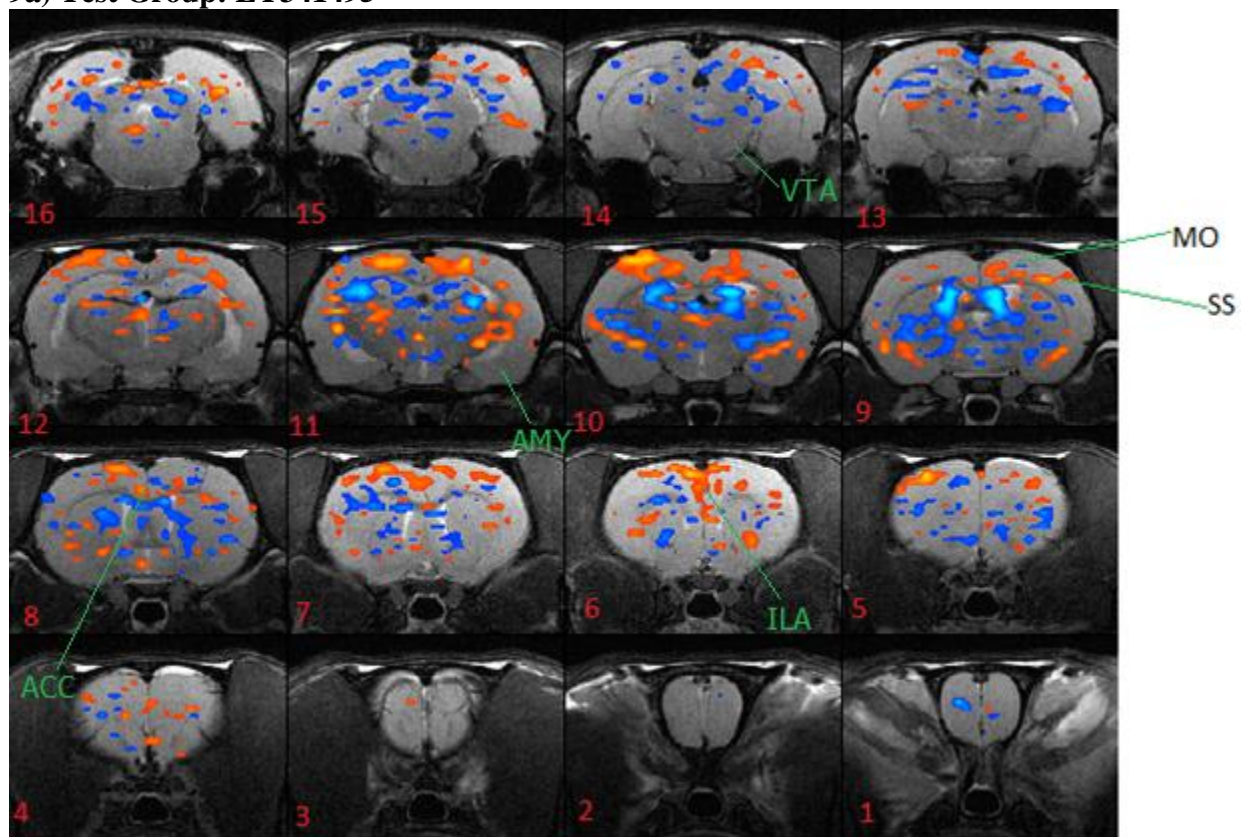
Figure 8a shows images of the control group in the nicotine-craving state after receiving saline. Previous studies have shown that exposure to cigarette/nicotine-related cues to those dependent – which leads to craving – results in increased activity of the mesolimbic system (right posterior amygdala, posterior hippocampus, VTA, and medial thalamus) (Due *et al*, 2002) as well as increased glucose metabolism in parts of the anterior paralimbic system (anterior cingulate cortex (ACC), posterior orbitofrontal cortex (OFC), and anterior insula), dorsolateral prefrontal cortex (DLPFC) and right superior sensorimotor cortex (Brody *et al*, 2002). Thus, the saline scan was expected to show increased BOLD activity in parts of the mesolimbic and anterior paralimbic systems as well as in the DLPFC and right superior sensorimotor cortex. Seen in images 9 and 10 of Figure 8a, there is activation in the somatomotor (MO) and somatosensory (SS) areas. However, there does not seem to be much activation in other expected areas (Figure 8a): amygdala (AMY) (image 11), VTA (image 14), and the ACC (image 8) – though other regions such as the hippocampus, thalamus, OFC, anterior insula, and DLPFC cannot be as clearly identified from the imaging results.

After the 30 minute saline scan, rats received nicotine and underwent another 30 minute EPI scan. Since nicotine, similar to other drugs of abuse, is known to affect the mesocorticolimbic dopaminergic pathway, which is associated with the reward pathway in the

brain, positive BOLD activity was expected to be seen in the VTA, nucleus accumbens, amygdala, and prefrontal cortex (Xi *et al*, 2009). Figure 8b shows the imaging results after nicotine administration. Comparing Figures 8a and 8b, increased activation can be found in both the ACC and infralimbic area (ILA) seen in images 4-8, as well as a slight increase in activation of the retrosplenium (RSP) seen in images 9-10. However, there now seems to be decreased activation in the somatomotor and somatosensory areas (compare images 9 and 10 between Figures 8a and 8b). Furthermore, components of the reward pathway – the VTA (image 14 of Figure 8b), nucleus accumbens (image 5 of Figure 8b), and amygdala (image 11 of Figure 8b) – did not show much activation either after nicotine administration.

Since nicotine sensitization results in increased locomotor activity in rats (Balfour *et al*, 1998), positive BOLD responses were also speculated to be seen in components of the motor loop, more specifically, in the basal ganglia (caudate nucleus, putamen, globus pallidus, subthalamic nucleus, and substantia nigra). However, there does not seem to be much general activation of the basal ganglia – though specific components of this region cannot be as clearly identified from the imaging results. Overall, when comparing the saline scan to nicotine scan, aside from the already mentioned differences in brain activity, there does not appear to be a significant increase or decrease in BOLD response - though slight decreases in response can be seen in different brain areas in images 8, 10, and 14 of Figure 8b.

9a) Test Group: LY341495



9b) Test Group: Nicotine

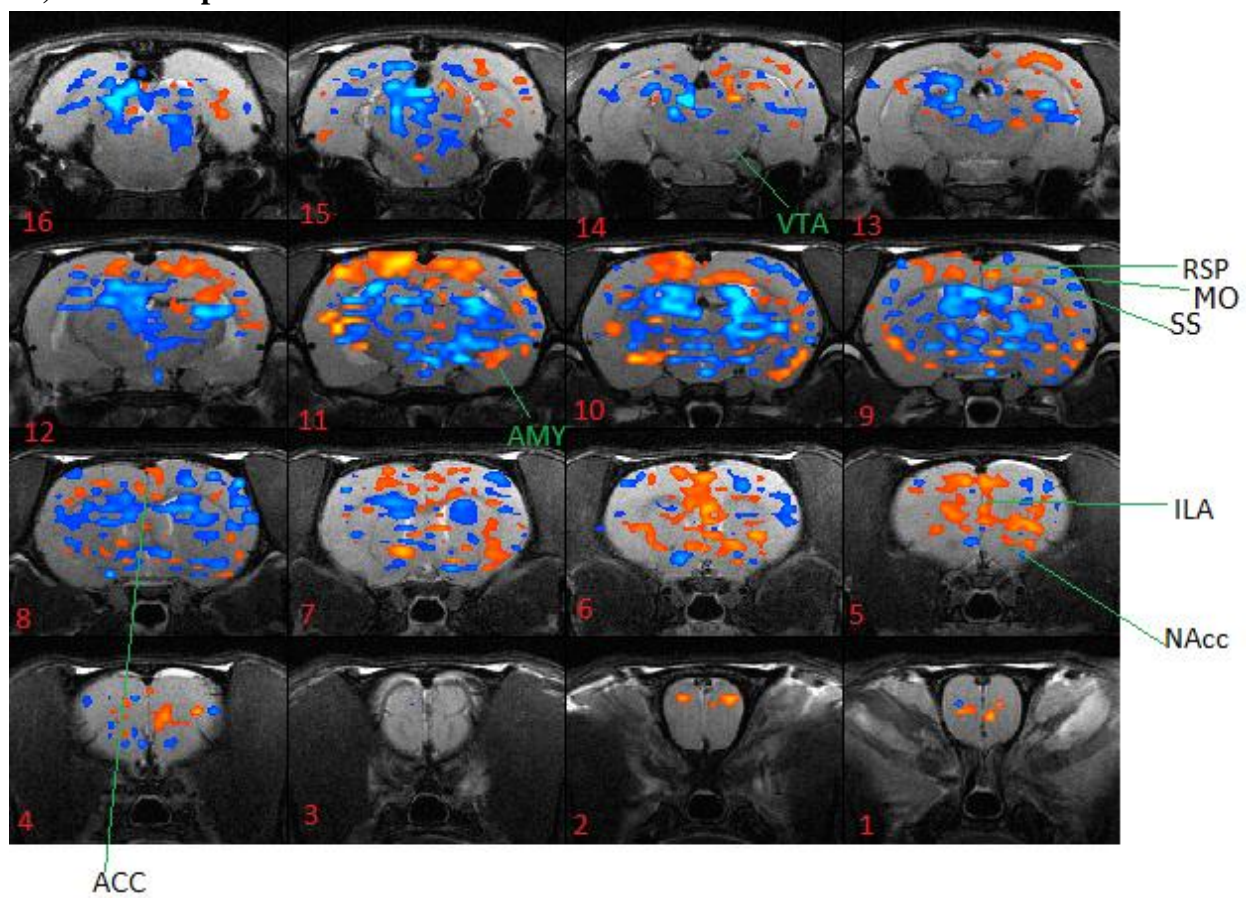


Figure 9: BOLD Response from LY341495-Treated Rats ($n=2$)

After the initial anatomical scan, two 30 minute EPI scans were performed (after injection of LY341495 and then after nicotine). The 16 images are brain slices from the forebrain to the hindbrain in numerical order. (MO = somatomotor area, SS = somatosensory area, ACC = anterior cingulate cortex, NAcc = nucleus accumbens, ILA = infralimbic area, RSP = retrosplenium, AMY = amygdala, VTA = ventral tegmental area)

The imaging procedure for the treatment group (receiving LY341495) was the same as that of the control group but LY341495 was administered instead of saline in the first EPI scan. Figure 9a shows images obtained after subjects received LY341495, and similar to the control group (Figure 8a), the rats were craving nicotine at this point. As a whole, the scans from the treatment group (Figure 9a) show more scattered positive BOLD responses compared to the scans from control group (Figure 8a), which show more concentrated positive BOLD responses. In addition, there are more negative BOLD responses in Figure 9a compared to Figure 8a – seen prominently in images 9-16. In contrast to the saline scan (Figure 8a), there does not seem to be significant activation of the somatomotor or somatosensory areas (image 9 of Figure 9a) in the LY341495 scan. However, there appears to be slight increased activation of the infralimbic area seen in image 6 (compare Figures 8a and 9a). Moreover, similar to the saline scan, there does not seem to be much activation in the other areas that are linked to nicotine craving (Figure 9a): amygdala (image 11), VTA (image 14), and the ACC (image 8) – other regions such as the hippocampus, thalamus, OFC, anterior insula, and DLPFC cannot be as clearly identified from the imaging results.

Figure 9b shows the results of the nicotine scan for LY341495-treated subjects. Comparing the LY341495 (Figure 9a) and nicotine (Figure 9b) scans, there does not appear to be an overall increase in positive BOLD response – though there are slight increases in brain regions seen in images 1 and 2, 5 and 6 (increased response in the infralimbic area), as well as 11

(increased response in somatomotor and somatosensory areas). In fact, there seems to be a general decrease in BOLD response between Figures 9a and 9b, seen prominently in images 8-12 and 15-16. However, there does appear to be slight activations in components of the reward pathway seen in the nucleus accumbens (image 5 of Figure 9b) and amygdala (image 11 of Figure 9b) but no apparent activation of the VTA (image 14 of Figure 9b). Similar to the nicotine scan for the control group, there does not seem to be activation in the basal ganglia – though specific components of this brain region cannot be as clearly identified from the imaging results. On the other hand, a decrease in BOLD response can be seen in the ACC, infralimbic area, and retrosplenium when comparing the treatment group (Figure 9b) with the control group (Figure 8b) (images 5-10). These results suggest that there is decreased corticolimbic activity between the control and treatment groups, and the decrease in BOLD response seen in the treatment group's nicotine scan (Figure 9b) may be attributable to the effects of LY341495.

Discussion

Of the 51 known carcinogenic chemicals in cigarettes, nicotine is the main component that leads to addiction (Markou, 2007). Extensive studies have been performed on the relationship between nicotine and dopamine, but focus is now being turned to another neurotransmitter that works closely with the dopamine system – glutamate. When nicotine enters the brain, it can bind to nAChRs on dopaminergic neurons to directly stimulate the release of dopamine into the nucleus accumbens, or nicotine can bind to the nAChRs on glutamatergic neurons to trigger the release of glutamate, which then results in the release of more dopamine (see Figure 1) (Markou, 2008). The present study focused on the effects of blocking Group II metabotropic glutamate receptors in nicotine-sensitized rats with the use of the novel mGluII receptor antagonist LY341495.

Open-field test

The effects of LY341495 on locomotor activity were measured by an open-field test. Since LY341495 was believed to prevent the inhibitory activity of the mGluII receptors, an increase in glutamate transmission was predicted to occur, followed by a rise in dopamine transmission in nicotine-sensitized rats. An increase in the level of dopamine in the terminal regions of the mesolimbic system – which projects mainly from the VTA to the nucleus accumbens – is thought to be responsible for the psychomotor-stimulant effects of nicotine (Clarke *et al*, 1988; Louis and Clarke, 1998; Pontieri *et al*, 1996). However, the initial dose of nicotine is known to cause a decrease in locomotor activity in nicotine-naïve rats. After repeated doses, though, the subjects become sensitized to the effects of nicotine; that is, they develop

tolerance to its depressant effects (Collins *et al*, 1988) and sensitization to its stimulant effects (Clarke and Kumar, 1983a; Ksir *et al*, 1985). As a result, their locomotor activity will increase, which is believed to be caused by the stimulation of dopamine neurons (Balfour *et al*, 1998). Since LY341495 is predicted to increase glutamate transmission by blocking the inhibitory effects of mGluII receptors and thus, augment the effects of nicotine, nicotine-sensitized rats treated with LY341495 in the present study were predicted to exhibit an increase in locomotor activity. However, results from the open-field test did not support this hypothesis.

Seen in Figure 7, both the control and LY341495-treated groups showed the expected initial drop in locomotor activity from Day 0 (baseline) to Day 1. Both groups also exhibited a general gradual increase in mean locomotor activity from Days 1-7, but the control group had higher values for all those days – significantly higher on Days 6 and 7. While the control group seemed to have reached a plateau on Days 6 and 7 – when subjects were given either saline or LY341495 – the treatment group still exhibited a gradual increase in mean locomotor activity. Though the slight increases followed the overall increasing trend established from the previous days, LY341495 did not appear to have a significant effect on locomotor activity. The difference in activity between Day 5 and 6 was only approximately 260 cm and the difference between Day 6 and 7 was approximately 100 cm (see Appendix for raw data). LY341495-treated group never reached the control group's mean activity on Days 6 and 7. If the initial hypothesis were true, the mean activity for the treatment group should be much closer, if not higher, than the values for the control group on Days 6 and 7, despite the fact that the treatment group showed lower activity compared to the control group on all days except for Day 0 (baseline).

No outliers could be identified from the raw data on locomotor activity due to the high standard deviations for both groups on each day. However, one of the rats from the treatment

group seemed to generally exhibit less locomotor activity compared to the others within the same group (Rat 1 of LY341495-treated group), but the difference was not significant enough to deem this subject as an outlier. Moreover, the behavioral tests were performed at the same time everyday for both groups, so Circadian rhythm should not have an effect on the results. The subjects' test performance, dealing with learning and memory processes, in the present study should not have been affected by LY341495 either because the mGluII receptor antagonist actually improved spatial learning in mice in the Morris water maze (Higgins *et al*, 2004).

The treatment group's low level of locomotor activity might be attributable to increased anxiety in the rats. Previous studies have revealed that LY341495 increased anxiety-like behavior in mice measured by the elevated-plus maze (Linden *et al*, 2005). Contrary to the initial hypothesis, LY341495 has also been shown to have no effect on locomotor activity: in a study where nicotine was not involved, rats that received microinjections of the drug into their nucleus accumbens did not exhibit any differences in locomotor activity (Richard and Berridge, 2010).

Another factor that may influence the results of this study is the dosage of LY341495. O'Neill *et al*. observed the locomotor activity of mice after given LY341495 – no nicotine was involved in this study. A range of doses were tested and results showed that hyperactivity was produced at the minimum effective dose of 2.5 mg/kg, while 1 mg/kg (dosage in the present study) did not seem to have much effect (O'Neill *et al*, 2003).

fMRI

In order to observe the effects of LY341495 on brain activity, a new batch of rats were used and treated to the same drug protocol as the subjects in the behavioral study (open-field test). (Note: Images of the control group were obtained from a similar nicotine study that used a different drug but employed the same control protocol as the present study.) It is important to note that there were only 2 rats in the treatment group (there was an initial $n = 4$) while there were 4 in the control group. Due to technical difficulties with the fMRI magnet, one of the rats in the treatment group could not be used for the study because it was in the scanner for too long during the repair process; and thus, the results would have been affected since the rat would have been too stressed. In addition, images from another subject were not as clear or well-defined as desired and were omitted from the analysis.

Studies have shown that exposure to cigarette/nicotine-related cues to people dependent on the drug will lead to craving and subsequent increase in activity of the mesolimbic system (right post posterior amygdala, posterior hippocampus, VTA, and medial thalamus) (Due *et al*, 2002). Increased glucose metabolism in parts of the anterior paralimbic system (ACC, posterior OFC, and anterior insula), DLPFC, and right superior sensorimotor cortex (Brody *et al*, 2002) are also expected. Since both the control group's saline scan and the treatment group's LY341495 scan preceded the nicotine scan in our study, the rats were expected to be in the craving state at this point. Thus, positive BOLD responses were predicted in the mesolimbic system and parts of the anterior paralimbic system as well as in the DLPFC and right superior sensorimotor cortex. However, as seen in Figures 8a and 9a, vital regions associated with nicotine craving did not show significant activation: amygdala (image 11), VTA (image 14), and the ACC (image 8). Brain regions such as the hippocampus, thalamus, OFC, anterior insula, and

DLPFC cannot be as clearly identified from the imaging scans, but there does not appear to be discernable activation in those regions.

Though both pre-nicotine scans did not show much activation in majority of the brain regions associated with nicotine craving, the saline scan did show activation in the somatomotor and somatosensory areas (image 9 of Figure 8a), while the LY341495 scan displayed decreased activation in those regions (image 9 of Figure 9a). The decreased BOLD response seen in the treatment group could possibly be due to LY341495's influence in blocking the effects of nicotine – which would also inhibit craving. However, this would not account for the increased activation of the infralimbic area seen in those treated with LY341495 (image 6 of Figure 9a) compared to the control (image 6 of Figure 8a).

Similar to other drugs of abuse, nicotine is known to affect the reward pathway (Xi *et al*, 2009). Thus, positive BOLD response was expected to be seen in the VTA, nucleus accumbens, and amygdala in the nicotine scans for both groups. In the control group's nicotine scan (Figure 8b), there did not seem to be activation in any of those three brain regions (images 14, 5, and 11 respectively). However, there appeared to be slight activation in the nucleus accumbens (image 5) and amygdala (image 11) in the treatment group's nicotine scan (Figure 9b) but no significant activity in the VTA (image 14). On the other hand, a decrease in BOLD response can be seen in the ACC, infralimbic area, and retrosplenium when comparing the treatment group (Figure 9b) with the control group (Figure 8b) (images 5-10). In general, there is decreased corticolimbic activity in the treatment group after the administration of nicotine (Figure 9b) compared to the control group after receiving nicotine (Figure 8b).

In animal studies, nicotine sensitization results in an increase in locomotor activity (Balfour *et al*, 1998). Therefore, the motor loop was thought to possibly be affected by both

nicotine and LY341495, and positive BOLD responses were expected in the basal ganglia: caudate nucleus, putamen, globus pallidus, subthalamic nucleus, and substantia nigra. However, the LY341495 (Figure 9a) and both nicotine scans (Figures 8b and 9b) did not show significant activation of the basal ganglia – though the specific brain components could not be as clearly identified from the imaging results.

As mentioned earlier, there appears to be activation in the somatomotor and somatosensory areas (images 9 and 10 of Figure 8a) in the saline scan. Interestingly, there was decreased activation in those two areas after the administration of nicotine (images 9 and 10 of Figure 8b) but increased activity in the ACC and the infralimbic area (images 4-8 of Figures 8a and 8b), as well as slight increased activation in the retrosplenium (images 9 and 10 of Figures 8a and 8b). Though the exact functions of the ACC are still debated, research has implicated the involvement of this brain region in conflict and error monitoring (Carter *et al*, 1998; Falkenstein *et al*, 2000), depression and anxiety disorders (Mayberg *et al*, 2000; Brody *et al*, 2001), and even pain perception (Rainville *et al*, 1997). In human functional imaging studies, the ACC has been activated during periods of anxiety – increased activation related to greater anxiety and decreased activation related to depressed mood (Kimbrell *et al*, 1999; Chua *et al*, 1999) – alertness (Sturm *et al*, 1999; Naito *et al*, 2000), arousal (Rauch *et al*, 1999; Stoleru *et al*, 1999; Critchley *et al*, 2001), focused attention (Bench *et al*, 1993; Keilp *et al*, 1997; Davis *et al*, 1997; Benedict *et al*, 1998; Woldorff *et al*, 1999), and awareness of emotional state (Lane *et al*, 1998). On the other hand, human studies have shown that the retrosplenium is responsible for episodic memories and navigation (Vann *et al*, 2009), while extinction memory of drug rewards, such as cocaine, has shown the involvement of the infralimbic cortex (Peters *et al*, 2008).

When comparing the results from the treatment group (Figure 9a and 9b), slight increases in activity can be seen in the somatomotor and somatosensory areas (image 11) as well as in the infralimbic area (images 5 and 6) after the administration of nicotine. However, there was an overall decrease in BOLD response seen after the nicotine injection. This phenomenon could be attributed to LY341495's ability to block the effects of nicotine – though this would not account for the increased activation of the nucleus accumbens (image 5) and amygdala (11) but inactivity of the VTA (image 14).

Aside from the already mentioned plausible explanations for the differences in brain activity observed between the control and treatment groups, another factor affecting the results is the sample size. As mentioned earlier, the images of the treatment group were from only 2 out of the original 4 subjects. Therefore, more rats need to be added to the treatment group in order to delineate statistically significant effects of LY341495. The treatment group's scans (Figure 9) also show scattered BOLD responses and a significant amount of negative BOLD activity when compared to the scans from the control group (Figure 8). Despite performing motion artifact correction in Matlab, these results could have possibly been due to motion. Hence, the resulting images for the treatment group may not show an accurate depiction of the subjects' brain activity.

Conclusion

The present study evaluated the role of glutamate in nicotine sensitization through behavioral tests and imaging (fMRI). The novel Group II metabotropic glutamate receptor antagonist LY341495 was administered to rats and their locomotor and brain activities were assayed. Results from the open-field test did not support the initial hypothesis that LY341495

would augment the effects of nicotine in sensitized rats, since LY341495-treated rats did not show an increase in locomotor activity compared to the controls. In addition, brain images (fMRI) from both the control and treatment groups were not as expected either since there was a lack of brain activity in regions associated with craving (pre-nicotine scans) and motor activity (after nicotine). No activation was seen in the reward pathway after the administration of nicotine in the control group, but there may have been slight activity in the amygdala and nucleus accumbens in the treatment group.

To further investigate the effects of LY341495 and possibly elucidate the current results, the study could be repeated with a different dosage of LY341495. As mentioned earlier, O'Neill *et al.* found the minimum effective dose to produce hyperactivity to be 2.5 mg/kg. Though nicotine was not involved in that experiment, raising the dosage of LY341495 in the current study (1 mg/kg) could possibly yield different patterns in both behavioral and imaging assays.

On the other hand, the rats may not have been in the chronic nicotine state at the time of testing. In the present study, the subjects received subcutaneous injections of nicotine (0.4 mg/kg) for 5 consecutive days before receiving the treatment (LY341495) or saline. However, other studies that measured the effects of nicotine on locomotor activity have employed more days of nicotine treatment: subcutaneous nicotine injections were administered for 37 consecutive days (0.2 or 0.4 mg/kg) by Ericson *et al.* (Ericson *et al.*, 2010) and for 49 consecutive days (0.4 mg/kg) by Clarke and Kumar (Clarke and Kumar, 1983). To our knowledge, this is the first study to examine the effects of LY341495 through fMRI. This study should be repeated with an increased dosage of LY341495, as well as a larger sample size for imaging, in order to yield more accurate and statistically significant results.

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Appendix

Treatment Group (LY341495) Locomotor Activity Raw Data

(Distance is measured in cm and ‘#2’ denotes Batch #2)

	Rat 1	Rat 2	Rat 3	Rat 4	Rat 1 (#2)	Rat 2 (#2)	Rat 5 (#2)	Rat 6 (#2)
Baseline	12267.88	11334.56	11813.49	11275.57	12455.04	9900.54	9827.26	13224.7
Day 1	5340.55	4025.87	3692.8	7221.23	8008.81	3018.2	5533.65	7688.7
Day 2	4299.55	5780.79	8092.58	7703.88	8951.57	4988.47	7502.21	9218.33
Day 3	3732.6	7203.07	5462.02	9502.06	10759.9	7374.22	7677.57	10415.68
Day 4	6284.87	9776.57	9443.68	10922.95	12923.39	6399.29	9774.33	10535.54
Day 5	4891.74	9356.24	9404.05	10378.85	14079.56	7928.5	10845.71	11094.13
Day 6	7756.21	10436.41	10421.81	10853.93	12479.07	8403.2	9736.52	9968.85
Day 7	7515.27	10633.67	11221.86	10882.31	13942.52	10002.87	12761.9	11186.18

Control Group (Saline) Locomotor Activity Raw Data

(Distance is measured in cm and ‘#2’ denotes Batch #2)

	Rat 5	Rat 6	Rat 7	Rat 8	Rat 3 (#2)	Rat 4 (#2)	Rat 7 (#2)	Rat 8 (#2)
Baseline	10291.02	12571.88	9744.69	9035.02	10914.88	10625.22	14480.28	12950.71
Day 1	6535.88	6699.21	5950.66	2068.45	6435.15	6262.92	7828.23	7166.76
Day 2	7421.01	9617.35	7090.39	4334.21	8209.5	5452.42	10418.22	9141.69
Day 3	10049.81	12624.25	8633.38	7073.36	10305.95	6993.3	11609.15	10378
Day 4	11592.66	12633.79	10963.74	7096.06	12538.03	6291.48	13580.83	12695.31
Day 5	13476.93	14898.27	12683.01	7446.76	11576.74	9325.52	15867.14	12676.96
Day 6	13288.66	17187.31	12398.48	9179.41	15407.67	11403.15	16871.97	15831.2
Day 7	13941.92	18154.34	12705.36	10825.37	15728.1	11588.48	15520.18	13723.85

Calculated Mean and Standard Deviations

(Yellow-highlighted rows are calculations for treatment group while white/non-colored rows are for control group.)

	Mean	Std Dev	Mean	Std Dev
Baseline	11512.38	1195.674	11326.71	1836.993
Day 1	5566.226	1913.747	6118.408	1735.58
Day 2	7067.173	1830.693	7710.599	2078.177
Day 3	7765.89	2421.717	9708.4	2018.058
Day 4	9507.578	2233.596	10923.99	2733.047
Day 5	9747.348	2654.996	12243.92	2778.717
Day 6	10007	1458.851	13945.98	2847.837
Day 7	11018.32	1894.713	14023.45	2396.451

ANOVA: Single Factor (p < 0.05)

Between Groups

Day 1

SUMMARY				
Groups	Count	Sum	Average	Variance
Column 1	8	44529.81	5566.226	3662429
Column 2	8	48947.26	6118.408	3012236

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1219617	1	1219617	0.365447	0.555168	4.60011
Within Groups	46722655	14	3337333			
Total	47942272	15				

Day 2

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Column 1	8	56537.38	7067.173	3351438
Column 2	8	61684.79	7710.599	4318819

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	1655989	1	1655989	0.431795	0.521766	4.60011
Within Groups	53691796	14	3835128			
Total	55347785	15				

Day 3

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Column 1	8	62127.12	7765.89	5864712
Column 2	8	77667.2	9708.4	4072556

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	15093380	1	15093380	3.037732	0.10326	4.60011
Within Groups	69560881	14	4968634			
Total	84654262	15				

Day 4

SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Column 1	8	76060.62	9507.578	4988950
Column 2	8	87391.9	10923.99	7469544

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	8024869	1	8024869	1.288257	0.275429	4.60011
Within Groups	87209464	14	6229247			
Total	95234333	15				

Day 5

SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Column 1	8	77978.78	9747.348	7049005
Column 2	8	97951.33	12243.92	7721267

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	24931422	1	24931422	3.375892	0.087472	4.60011
Within Groups	1.03E+08	14	7385136			
Total	1.28E+08	15				

Day 6

SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Column 1	8	80056	10007	2128247
Column 2	8	111567.9	13945.98	8110176

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	62062293	1	62062293	12.12341	0.003665	4.60011
Within Groups	71668955	14	5119211			
Total	1.34E+08	15				

Day 7

SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Column 1	8	88146.58	11018.32	3589939
Column 2	8	112187.6	14023.45	5742978

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	36123165	1	36123165	7.741023	0.014684	4.60011
Within Groups	65330422	14	4666459			
Total	1.01E+08	15				

Within Group (LY341495)

Day 5 vs. 6

SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Column 1	8	77978.78	9747.348	7049005
Column 2	8	80056	10007	2128247

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	269677.7	1	269677.7	0.058771	0.811965	4.60011
Within Groups	64240758	14	4588626			
Total	64510436	15				

Day 5 vs. 7

SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Column 1	8	77978.78	9747.348	7049005
Column 2	8	88146.58	11018.32	3589939

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	6461510	1	6461510	1.21469	0.288992	4.60011
Within Groups	74472607	14	5319472			
Total	80934117	15				